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The effects of foliar application of ascorbic acid (vitamin C) on physiological and biochemical changes of corn (*Zea mays* L) under irrigation withholding in different growth stages

Mahmoud Darvishan[1], Hamid R Tohidi-Moghadam[1]*, Hossein Zahedi[2]

[1]Department of Agronomy, Varamin-Pishva Branch, Islamic Azad University,Varamin, Iran
[2]Department of Agronomy and Plant Breeding, Eslamshahr Branch, Islamic Azad University,Tehran, Iran
*Corresponding author: E-mail: hamid_tohidi2008@yahoo.com

Abstract

To study the effects of ascorbic acid foliar application and limited irrigation in different growth stages on physiological and biochemical changes of corn, an experiment was conducted in Varamin, Iran during the growing season of 2012. The experimental design was randomized complete blocks in split plots arrangement with three replications. Main plots included four different levels of irrigation (complete irrigation, irrigation withholding at 8-leaf stage, irrigation withholding at silks appearance stage and irrigation withholding at both 8-leaf stage and silks appearance stages) and different concentration of foliar application of ascorbic acid (vitamin C) (0, 75, and 150 ppm) were allocated to subplots. The results showed that irrigation withholding conditions in different growth stages significantly decreased seed yield, RWC and total chlorophyll but by contrast increased proline content, antioxidant enzymes activity. Ascorbic acid foliar application in irrigation with holding in different growth stages had positive effect on all attributes in this experiment. In general, the results of the present study indicate that usage of AsA reduces the harmful effects of water deficit stress and increases resistance to drought stress in corn plant.

Keywords: antioxidant enzymes activity, relative water content, chlorophyll

Abbreviations: RWC - relative water content, AsA - ascorbic acid, ROS - reactive oxygen species, CAT - catalase, SOD - Superoxide dismutase, GPX - Glutathione peroxidase, Pro - proline, Chl - Chlorophyll

Introduction

Today, across the globe, corn is a direct staple food for millions of individuals and, through indirect consumption as a feed crop, is an essential component of global food security (Campos et al, 2004). In many regions of the world, including Iran, drought stress is one of the most important factors responsible for decreasing crop yield. Identification of the critical irrigation timing and scheduling of irrigation based on a timely and accurate basis to the crop is the key to conserving water and improving irrigation performance and sustainability of irrigated agriculture (Ngouajio et al, 2007). Water stress induces oxidative stress in plants (Hajiboland and Joudmand, 2009). Zahedi et al (2011) reported that antioxidant enzymes activity were increased when plants were exposed to water stress. Under conditions of water stress and other types of environmental stress, reactive oxygen species (ROS), such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals, are generated (Zhu, 2000). Plant cells contain an array of protection mechanisms and repair systems that can minimize the occurrence of oxidative damage caused by reactive oxygen species (ROS) (Abdel Latef, 2010). The induction of ROS-scavenging enzymes, such as superoxide dismutase, catalase, peroxidase, and ascorbate peroxidase is the most common mechanism for detoxifying ROS synthesized during stress response (Gressel and Galun, 1994). In the antioxidants system, which involves antioxidant substances such as tocopherols and AsA (Foyer et al, 1994). This last compound is a small, water-soluble antioxidant molecule, that acts as a primary substrate in the cyclical pathway for detoxification and neutralization of superoxide radicals and singlet oxygen (Noctor and Foyer, 1998). It has also been reported that application of exogenous ascorbate can increase resistance to salt stress and reduce oxidative stresses (Shalata and Neumann, 2001). Ascorbate has been shown to play multiple roles in plant growth, such as in cell division, cell wall expansion, and other developmental processes (Pignocchi and Foyer, 2003). The work was aimed also whether a foliar supply of AsA to plant might be a strategy for increasing the water deficit tolerance.

Materials and Methods

The experiment was conducted in an experimental field area of the Azad University, Varamin Branch in Iran during 2012 growing season. The site of study was situated at 31°519'E and 20°359'N and 1,050

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Table 1 - Soil properties of the experimental site.

Depth	EC (ds m^{-1})	pH	O.C (%)	T.N.V (%)	K (ppm)	P (ppm)	Total N (%)	Texture
0 - 30 cm	4.1	7.4	0.71	<10	368	25.9	0.079	Clay loam

masl. Latitude and longitude of the research place was 35°19'N and 51°39'E, respectively, and the site of study was located 900 masl. Before the beginning of the experiment, soil samples were taken to determine the physical and chemical properties. A composite soil sample was collected at a depth of 0 - 30 cm. It was air dried, crushed, and tested for physical and chemical properties. The experimental area had a clay loam soil. Details of soil properties are shown in Table 1. After plough and disk, plots were prepared. The experimental design was randomized complete blocks in split plots arrangement with three replications. Main plots included four different levels of irrigation (I1: complete irrigation, I2: irrigation withholding at 8-leaf stage, I3: irrigation withholding at silks appearance stage, and I4: irrigation withholding at both 8-leaf stage and silks appearance stages). Different concentration of foliar application of ascorbic acid (vitamin C) was allocated as subplots (S1: 0, S2: 75, and S3: 150 ppm). The elementary plots were 5 m long and consisted of five rows, 0.75 m apart. Between all main plots, a 2 m alley was kept to eliminate all influence of lateral water movement. Polyethylene pipeline was performed for control of dropping irrigation. Treflan and Gallant super were applied to control weeds. According to soil analysis, phosphorus (150 kg ha^{-1}) and potassium (200 kg ha^{-1}) fertilizers were applied to the soil. Nitrogen was supplied from ammonium nitrate (300 kg ha^{-1}) at three stages; seed sowing, 8-leaf stage and before flowering stage. The plots were sown on May 2012 by hands seeds to have a distance of 20 cm between plants. Two seeds were sown on May 2012, 20 cm apart along the rows, the plots were thinned to the desired plant population (67,000 plant ha^{-1}). After seed sowing, irrigation was applied as required during the growing season. The foliar application of AsA was applied with a pressurized back pack sprayer calibrated to deliver 1000 l ha^{-1} of spray solution. Sprayer was equipped with a spiral solid cone spray nozzle. At the end of growing season crop were harvested and seed yield was assayed. One week after irrigation withholding in different growth stages initiation leaf samples were collected and physiological and biochemical changes were assayed.

Relative water content assay

Relative water content (RWC) was measured, from each plant leaf discs were taken and weighted (fresh weight, FW). The discs were then placed in distilled water for 5 h at 25°C and then their saturated weights (SW) were measured. The discs were then dried in oven at 70°C for 24 h to calculate dry weight (DW). Relative water contents were calculated by following formula (Gupta, 2000):

RWC= (FW - DW)/(SW - DW)

Chlorophyll assay

The first fully expanded leaf blades were taken to determine chlorophyll (Chl) contents. For the chlorophyll assay, leaf discs were ground with 10 ml of 80% acetone (v/v). The amount of chlorophyll a and b was determined spectrophotometrically at 663 and 645 nm, using the method of Arnon (1949).

Proline assay

Pro content of leaves was determined according to a modification of the method of Bates et al (1973). Samples of leaves (0.5 g) were homogenized in a mortar and pestle with 10 ml sulpho-salicylic acid (3% w/v), and then centrifuged at 18,000 g for 15 min. Two ml of the supernatant was then added to a test tube, to which 2 ml glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) were added. The test tubes were incubated in a water bath for 1 h at 100°C and then allowed to cool to room temperature. Four ml of toluene were then added to the tubes and then mixed on a vortex mixer for 20 s. The test tubes were allowed to stand for at least 10 min, to allow separation of the toluene and aqueous phases. The toluene phase was carefully pipetted out into a glass test tube and its absorbance was measured at 520 nm in a spectrophotometer. The content of proline was calculated from a standard curve.

Antioxidant enzyme activity assay

Catalase (CAT) activity was measured according to Chandlee and Scandalios (1984), with modification. The assay mixture contained 2.6 ml of 50 mM potassium phosphate buffer (pH 7), 0.4 ml of 15 mM H_2O_2 and 0.04 ml of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm.

Glutathione peroxidase (GPX) activity was measured according to Paglia and Valentine (1997) in which 0.56 M (pH 7) phosphate buffer, 0.5 M EDTA, 1mM NaN_3, 0.2 mM NADPH was added to the extracted solution. GPX catalyses the oxidation of glutathione by cumene hydroperoxide in the presence of glutathione reductase and NADPH, the oxidized glutathione is immediately converted to the reduced form with the concomitant oxidation of NADPH to NADP. The decrease in absorbance at 340 nm was measured with a spectrophotometer.

Superoxide dismutase (SOD) activity was assayed according to Beauchamp and Fridovich (1971). The

Table 2 - Analysis of variance on attributes of corn affected by irrigation withholding in different growth stages and foliar application of ascorbic acid.

S.O.V.	d.f.	Plant height	1000 seed weight	Seed yield	Relative water content	Total Chl	Pro	SOD	CAT	GPX	AsA
Replication	2	**	**	**	**	ns	ns	ns	ns	ns	ns
Water stress	3	**	**	**	**	**	**	**	**	**	*
Error (a)	6										
Ascorbic acid application	2	ns	**	**	**	**	**	**	**	*	**
Water stress × Ascorbic acid application	6	**	**	**	**	**	**	ns	ns	ns	ns
Error (b)	16										
C.V (%)		7.78	1.07	4.27	3.82	0.61	15.99	7.70	15.45	17.32	19.05

*, ** and ns: significant at 0.05, 0.01 probability level and not significant, respectively

reaction mixture contained 1.17×10^{-6} M of riboflavin, 0.1 M of methionine, 2×10^{-5} M of potassium cyanide (KCN) and 5.6×10^{-5} M of nitro blue tetrazolium salt (NBT) dissolved in 3 ml of 0.05 M sodium phosphate buffer (pH 7.8).Three ml of the reaction medium were added to 1 ml of enzyme extract. The mixtures were illuminated in glass test tubes by two sets of Phillips 40-W fluorescent tubes in a single row. Illumination was started to initiate the reaction at 30°C for 1 h. identical solutions that were kept under dark served as blanks. The absorbance was read at 560 nm in the spectrophotometer against the blank.

Ascorbic acid assay

AsA was extracted from 2 g shoot fresh material by 4% oxalic, then made up to known volume (100 ml) and centrifuged at 2,000 rpm for 5 min. Subsequently, 10 ml of 4% oxalic acid were added and titration was performed using 2,6-dichlorphenol-indophenol as described by Sadasivam and Manickamm (1996).

Statistical analysis

All data were analyzed from analysis of variance (ANOVA) using the GLM procedure in SAS (SAS Institute, 2002). Assuming that the residuals were random, homogenous and with a normal distribution about a mean of zero. Treatment means were compared using LSMEANS ($P < 0.05$).

Results and Discussion

ANOVA showed that the effects of irrigation withholding in different growth stages and foliar application of AsA were significant on all measured traits of experiment except plant height (Table 2). In addition, interaction between these two factors was significant for all traits studied in this experiment. Interaction of experimental factors (irrigation withholding in different growth stages × foliar application of AsA) was significant for all traits. As can be seen from Table 3, the highest plant height was obtained from complete irrigation. Irrigation withholding at silks appearance stage did not decrease plant height. Since corn has determinate growth and vegetative growth would be stopped with entering to reproductive stage (Li et al, 2000). Effect foliar application of AsA was not significant on plant height (Table 2 and 3). Moreover, the results showed that 1000 seed weight decreased as result of irrigation withholding at 8-leaf, silks appearance and irrigation withholding at both 8-leaf and silks appearance stages by 10.85%, 32.98%, and 36.42%, respectively, in comparison to complete irrigation treatment conditions. It is evident that irrigation withholding at different growth stages had a negative influence on source and sink relationships. These results are in agreement with those obtained by Unger (1992) and Yegappan et al (1982) who reported that the most important factor increasing seed weight is soil water content during grain filling period. Water stress has a detrimental effect on available assimilates during grain-filling by decreasing sink capacity which in turn leads to unfilled seeds and consequently to a low seed weight (Li et al, 2000). Our results showed that foliar application of AsA with 150 ppm improved 1,000-seed weight under irrigation withholding in different growth stages.

Our results have also indicated that there was significant difference among irrigation regimes on seed yield (Table 2 and 3). Seed yield decreased as result of irrigation withholding at both 8-leaf, silks appearance and irrigation withholding at both 8-leaf and silks appearance stages by 30%, 47.92%, and 59.94%, respectively, when compared with data from complete irrigation treatment. Seed yield increased as result of foliar application of AsA with 150 ppm by 11.79%, 28.17%, and 31.86% under irrigation withholding at both 8-leaf, silks appearance and irrigation withholding at both 8-leaf and silks appearance stages, respectively, when this treatments were compared with untreated foliar application of AsA in this condition (Table 3). Decrease in seed yield due to decrease in yield components, especially seed weight, has been previously reported by other researchers (e.g. Yegappan et al, 1982). Decrease in length of grain-filling period due to water stress is the main factor to decrease seed weight (Cantagallo et al, 1997). Furthermore, water deficit decreased seed yield by decreasing photosynthesis and seed number per ear (Unger, 1992). The results showed that irrigation withholding at different growth stages decreased RWC. RWC increased as result of foliar application of AsA with 150

Table 3 - The interaction effect between irrigation withholding in different growth stages and foliar application of ascorbic acid on attributes of corn.

Irrigation	Foliar Application of Ascorbic acid	Plant height (cm)	1,000- seed weight (g)	Seed yield (t ha^{-1})	Relative water content (%)	Total chlorophyll (mg g^{-1} FW)	Pro (μmol g^{-1} FW)
Complete Irrigation							
	Untreated (0 ppm)	236.02ab	333.96a	11.143a	80.35ab	36.65b	0.013ef
	Treated (75 ppm)	231.06b	332.43a	11.166a	79.80ab	36.71b	0.016def
	Treated (150 ppm)	238.98a	338.16a	11.866a	81.29a	37.33a	0.010f
Irrigation withholding at 8-leaf stage							
	Untreated (0 ppm)	182.63c	297.73bc	7.756c	63.85de	32.48e	0.023d
	Treated (75 ppm)	184.48c	300.90b	8.180bc	66.22d	32.99d	0.020de
	Treated (150 ppm)	183.44c	306.90b	8.793b	76.50bc	34.43c	0.020de
Irrigation withholding at silks appearance stage							
	Untreated (0 ppm)	230.42b	223.80de	5.803d	56.70f	27.35h	0.040b
	Treated (75 ppm)	232.00b	233.43d	6.250d	60.03ef	29.42f	0.030c
	Treated (150 ppm)	233.20b	285.36c	8.080bc	72.80c	32.75de	0.020de
Irrigation withholding at both 8-leaf and silks appearance stages							
	Untreated (0 ppm)	181.08c	212.33e	4.463e	47.58g	26.22i	0.046a
	Treated (75 ppm)	183.98c	221.96de	4.946e	49.17g	27.94g	0.033c
	Treated (150 ppm)	180.48c	283.00c	6.550d	66.60d	32.50e	0.020de

Treatment means followed by the same letter within each common are not significantly different ($P < 0.05$) according to Duncan's Multiple Range test

ppm when these treatments compared with untreated foliar application of AsA in this condition (Table 3). RWC decreased as result of irrigation withholding at different growth stages. It was already reported that RWC is an appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit, while water potential is an estimate of plant water status and it is useful in dealing with water transport in the soil-plant-atmosphere continuum (Kramer, 1988). Foliar application of AsA with 150 ppm concentration increased RWC compared with untreated foliar application of AsA in irrigation withholding condition. Our results also indicated that foliar application of AsA with 150 ppm concentration decreased electrolyte leakage in irrigation withholding condition.The highest total chlorophyll content was related to those plots which were treated with complete irrigation. However irrigation withholding at different growth stages decreased total chlorophyll content. It is worth mentioning that total chlorophyll content increased as result of foliar application of AsA with 150 ppm when these treatments compared with untreated foliar application of AsA in this condition (Table 3). Chlorophyll content in plants is an important factor in determining photosynthetic capacity. Decreased or unchanged chlorophyll level during drought stress has been observed in other species, depending on drought duration and severity (Rensburg and Kruger, 1994; Kyparissis et al, 1995; Zhang and Kirkham,1996; Jagtap et al,1998). Changes in leaf chlorophyll content with drought and heat injury may involve a severe chlorophyll photo-oxidation mediated by oxy-radicals (Wise and Naylor,1987). Water deficit stress leads to an increase in free radicals in chloroplasts and destruction of Chl molecules by ROS; this results in reduction of photosynthesis and growth. Singlet oxygen atoms and O_2-radicals predominantly attack double bond-containing compounds (unsaturated fatty acids and Chl), thus damaging the chloroplast membrane system and photosynthetic reaction centre (Zhang et al, 2003), which, in turn, may result in the release of Chl from the thylakoid membranes. In such cases, the Chl needs to be degraded quickly to avoid cellular damage by its photodynamic action (Takamiya et al, 2000). AsA is a detoxifier and neutralizer of superoxide radicals and other singlet oxygen species; by prevention of the activity of free radicals it can enhance the Chl content. The result showed that the highest Pro content in the leaves of plants belong to untreated foliar application of AsA treatment in irrigation withholding at both 8-leaf and silks appearance stages. However, foliar application of AsA acid with 150 ppm decreased Pro content in the leaves of plants. The accumulation of Pro, a highly water-soluble amino acid, is a common metabolic response of plants to adversity. Therefore Pro has become an indicator of adaptation to adversity because of its involvement in the resistant capability of plants (Gossett et al, 1994a). It was shown that Pro protects membranes against the adverse effect of high concentrations of ions and may also function as a compatible hydrotropic and as a hydroxyl radical5 scavenger (Kavikishor et al, 1995). In the current study, irrigation withholding at different growth stages increased leaf Pro content and application of AsA scavenged ROS and prevented biosynthesis of extra proline. The concentration of this metabolite usually increases in response to water deficit stress (Nandwal et al, 2000).

Plants under irrigation withholding at different growth stages showed a significant increase in SOD, CAT and GPX activity in the leaves compared with control plants (Table 4). Application of AsA was not associated with a significant difference in SOD, CAT and GPX activity in non-stressed plants; however, application of AsA decreased SOD, CAT and GPX activity in plants (Table 4). Additionally our results showed that there was no significant difference between different irrigation withholding levels on AsA (Table 4). Its concentration increased as result of foli-

Table 4 - Comparison of means on some attributes of corn affected by irrigation withholding in different growth stages and foliar application of ascorbic acid.

Treatment	SOD activity (u mg protein^{-1})	CAT activity (u mg protein^{-1})	GPX activity (u mg protein^{-1})	AsA activity (mg g^{-1} FW)
Irrigation				
Complete Irrigation	673.34c	151.03b	96.79c	0.425a
Irrigation withholding at 8-leaf stage	775.75b	210.11a	159.45b	0.373a
Irrigation withholding at silks appearance stage	858.55a	229.37a	186.82b	0.363a
Irrigation withholding at both 8-leaf and silks appearance stages	920.61a	235.66a	230.93a	0.390a
Ascorbic acid foliar application				
Untreated (0 ppm)	915.02a	235.55a	189.35a	0.316c
Treated (75 ppm)	800.70b	209.15a	168.58ab	0.391b
Treated (150 ppm)	705.47c	174.93b	147.58b	0.457a

Treatment means followed by the same letter within each common are not significantly different ($P < 0.05$) according to Duncan's Multiple Range test

ar application of AsA with 150 ppm when these treatments were compared with untreated foliar application of AsA (Table 4). In the present study, the plants exposed to water deficit stress showed a significant increase in SOD, CAT, and GPX activity in the leaves. The enzymes assayed are scavengers of free radical species. SOD converts one form of ROS (O_2) to another equally toxic one (H_2O_2). Hydrogen peroxide is converted to oxygen and water by CAT and POX, which use ascorbate as the hydrogen donor (Hegedus et al, 2001). Water deficit stress may also lead to stomata closure, which reduces CO_2 availability in the leaves and inhibits carbon fixation (Gossett et al, 1994b). Similar increases in the activities of these enzymes were reported in cotton cultivars subjected to salt stress (Rajguru et al, 1999). The increase in SOD activity was reported to enhance tolerance of basmati rice varieties (Singh et al, 2007). In our study, AsA decreased the activity of these enzymes probably by decreasing the amount of free radicals. AsA has been found to be loaded in the phloem of source leaves and is then transported to other tissues (Tedone et al, 2004). When AsA was applied to the leaves of plants, as in our study, there was an obvious decrease in SOD, CAT, and GPX activities in the leaves. A role of AsA in the ascorbate-glutathione cycle in mitochondria and peroxisomes has been described (Jimenez et al, 1997).

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